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PERSISTENTLY LOW NATURAL KILLER CELL ACTIVITY AND
CIRCULATING LEVELS OF PLASMA BETA ENDORPHIN: RISK FACTORS FOR
INFECTIOUS DISEASE

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Summary

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Beta endorphin levels were quantitated in plasma samples obtained from normal subjects (n=81, 37% males and 63% females, age range 18-45 years) as a component of a prospective study examining the relationship of illness morbidity to natural killer cell activity and psychological indices of stress. The present study was designed to test whether beta endorphin levels contributed additionally to the explanation of illness outcome variance. In the larger study, persistently low NK (LNK) activity was associated prospectively with higher illness morbidity. The findings reported here suggest that the observed LNK activity might be affected by circulating levels of plasma beta endorphin, as lower endorphin levels predicted the LNK pattern, which in turn, predicted higher illness morbidity.

There is strong evidence that neuroendocrine polypeptides can modify a variety of components of the immune response. For example, gamma-interferon production has been shown to be influenced by opioid peptides (1), and NK activity appears to be enhanced by both beta-endorphin (2,3) and met-enkephalin (4-7). Overall, however, the mechanisms by which opioid peptides stimulate lymphocytes have been the subject of much discussion and few studies (8).

The present study was part of a larger project of infectious disease risk in a cohort of community volunteers. As reported elsewhere (9), 36% of a sample of 106 subjects showed natural killer (NK) cell activity persistently below the group mean across three baseline serial assessments. Based on earlier work (10), we hypothesized that higher perceived stress and younger chronological age would predict higher illness morbidity over a six month follow-up period via their effects on this pattern of natural immunity. We tested this hypothesis through causal path modeling (11), to evaluate relationships among the predictor variables, to test the utility of a causal hypothesis, and to make inferences regarding causality among the variables.

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The best fit model for the data showed both a direct and an indirect association between age and upper respiratory illness (URI) morbidity (defined as days reporting influenza or other upper respiratory symptoms) over the follow-up period. Specifically, older age predicted lower URI morbidity; younger age was associated with the LNK pattern, which in turn, strongly predicted higher follow-up URI morbidity. Interestingly, the strongest relationship of all was a predictive association found for perceived stress and illness, with higher average baseline stress levels directly predicting more URI illness morbidity over the follow-up period.

In a subset of this sample (N=81), we were also able to obtain complete baseline and follow-up plasma beta endorphin values, and examine its association with peripheral natural immunity and infectious disease risk over the prospective study period. These data are the subject of this report.

METHODS

Subjects. - One hundred and six community volunteers, between the ages of 18 and 45, participated in this project. Approximately 63% of the sample were females, 90% were caucasian, and 60% of the sample were single. Subjects were excluded on the following bases: History of alcohol or drug abuse; history of psychiatric hospitalization; current use of prescribed psychoactive agents; medically documented chronic disease (e.g., cardiovascular disease, diabetes, cancer), or recent (within the past two weeks) or current acute infectious or other physical disease. Normal individuals were recruited from the university and local community through newspaper ads and other media announcements, and, after signing a consent form, were interviewed regarding current and past health history.

Procedures. Subjects were serially tested, at baseline, and then again two weeks and four weeks into the study. They were then assessed monthly (except for a weekly health record, described below), including an abbreviated physical exam at three and six months post-accrual. Subjects were paid for their participation, and these payments, along with phone prompts, minimized loss of data in this prospective study.

Psychological Measures. The perception of environmental stressors was assessed at baseline induction into the study, and then at biweekly or monthly intervals, as specified over the follow-up period. Specifically, the Hassles Scale (12) is a 117-item questionnaire in which respondents are instructed to indicate the occurrence of any items (e.g., misplacing things, troublesome neighbors, etc.) which have "hassled" them within a specified period of time (e.g, in the past week). Participants rated each hassle on a 3-point scale as having been "somewhat," "moderately" or "extremely" severe. From this information, three scores were created: 1) a frequency score, which is a simple count of the number of items checked, 2) an intensity score, which is the mean severity reported by the participant for all items checked, and 3), a severity score, a total severity rating across all items checked. The scale has high test-retest reliability, with test-retest correlations in our sample over a five-month period (between one and six month assessment) of .78 for frequency scores, .60 for intensity scores, and .77 for severity scores. More recent work (13) showed that the hassles scores were more strongly associated with somatic health than were life events scores. Hassles shared most of the variance in

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health that could be accounted for by life events, and, when the effects of life events were statistically removed, hassles and health remained significantly related. In a recent publication (10) describing baseline characteristics of the current sample, we reported that the perception of hassles severity, rather than the frequency count of stressful levels, was a significant predictor of persistently low levels of NK cytotoxicity, and thus mean severity scores over the three baseline assessments were entered here in the results reported below.

Laboratory Measures

Blood samples were drawn between the hours of 9:00 a.m. and 2:00 p.m. Seventy-five percent of the samples were drawn before noon. An examination revealed no significant difference in NK activity between morning and early afternoon samples ($t=4$, NS). All assays of immune cell function were performed on fresh mononuclear cell samples held overnight in culture medium at 4°C to ensure uniformity of measurement across samples. NK activity, leukocyte counts and lymphocyte subpopulations in the circulation (percent T cells, B cells, and NK cells) were measured at baseline and follow-up periods.

Natural Killer Cell Assay. The K562 erythroleukemia cell line was maintained in culture in RPMI 1640 medium supplemented with 10% v/v fetal bovine serum (FBS). Cells were subcultured as needed, and the cells in the log phase of growth were used for cytotoxicity assays (14). Lytic units were calculated according to the formula of Pross et al. (15). One lytic unit was defined as the number of effector cells, out of 10^7 effector cells, that were required to kill 20% of 5×10^3 target cells. The data variability of the NK cell assay was monitored as described by us recently (16).

Flow Cytometry. The cells were adjusted to 0.5×10^6 /ml in phosphate buffered saline (PBS)-0.1% sodium azide buffer and stained with fluorescein-or phycoerythrin-labeled monoclonal antibodies against various surface markers on human mononuclear cells. The monoclonal antibodies were purchased from Becton-Dickinson (Mountain View, CA) and included: Leu 4, Leu 19, Leu 11a, Leu 12 and isotype controls. The cells were incubated with monoclonal antibodies, which were pretitered to give optimal staining, for 15 min. at 4°C. The stained cells were washed twice with PBS-azide buffer and resuspended in 200 μ l of 1% paraformaldehyde in the same buffer for two-color flow-cytometry analysis in FACSscan.

B-Endorphin Measurements in Plasma. Separate blood samples were drawn into 13x100 mm Vacutainers containing EDTA. Immediately, 0.3ml Aprotinin was added to each tube (30 TIU/ml, Sigma Chemical Company, St. Louis, MO), the tubes were inverted several times, and then refrigerated briefly (30-60 min) for transport to the radioimmunoassay (RIA) lab. The tubes were centrifuged at low speed (2200 rpm) for 20 minutes, and the plasmas removed and stored at -70°C. Plasma samples (1 ml) were assayed for B-END using an extraction and radioimmunoassay procedure obtained from Incstar (C/N 46065; Stillwater, MN). All values are corrected for recovery in the extraction (recovery \geq 90%). The sensitivity of the RIA is 3 pmol/l; the coefficients of variation are 13.7% (within assay) and 18.1% (between assay). This assay shows less than 0.01% cross-reactivity with beta lipotropin, leucine and methionine enkephalins, and ACTH.

Health Records. All subjects were asked to complete weekly illness, as well as lifestyle inventory records, developed during our pilot work. They were instructed to record daily symptoms of infectious illness experienced, as well as the amount of sleep per night, daily exercise, and daily alcohol, drug, caffeine, and tobacco consumed. Females also indicated presence or absence of menses. These records were collected by mail on a monthly basis, and phone prompts were made for subjects who failed to mail in their weekly recording. Presence/absence of symptoms reflecting colds, influenza, pneumonia, cold sores, gum infections, mononucleosis, strep throat, and gastro-intestinal illnesses, plus the direct reporting of symptoms such as fever and sore throat, were included on the record. If on follow-up physical exam, subjects reported illness in the preceding period, and if they reported seeing a physician, physician records were obtained in order to corroborate self-report. For the 30 physician office visits during the follow-up period, there was a 97% agreement between reported symptoms on the daily health record and chart documentation of presenting symptoms by physician.

It is recognized that the measure used here for illness episodes has all the limitations associated with self-report. However, past and current research has demonstrated that such report correlates rather well with clinical ratings of illness (17-19), viral shedding (20,21), and biochemical indicators of infection (22).

For the purpose of the present study, illness variables examined included overall days of illness morbidity for a wide variety of problems, such as colds, flu, gum infections, gastro-intestinal or "stomach" flu, and fever reported over the six month follow-up period, as well as days reported with upper respiratory morbidity (e.g., symptoms of cold and influenza) over follow-up. There were no reported cases of pneumonia or infectious mononucleosis in our sample during the follow-up period, and hence, these categories were not included in the morbidity data. Further, strep throat was eliminated from the morbidity tabulation because the two cases that were reported were not corroborated by laboratory analysis. Sore throat was eliminated from health data because of the possibility of confounding with fatigue or other physical stress conditions. Finally, cold sores were also eliminated in these analyses because we wished to examine incidence and/or morbidity associated with acute discrete illness, and we considered herpes viral infections a periodically expressed, chronic condition, interesting in itself, but distinct from the acute illnesses being tabulated over the follow-up period of this study. We chose to focus on reported morbidity, rather than attempt to distinguish between incidence of specific categories of illness, because of the difficulty in determining the validity of self-reported differential diagnosis on the part of the subject. Thus, the total duration of illness morbidity, rather than incidence of discrete infectious episodes, was the health end-point of major interest reported here.

For the larger study (9), as well as the results reported here, a causal path modeling technique (11) was used to analyze the data. Path analysis allows for the description and testing of hypothesized sequences of events within prospective research designs. This technique was used here to evaluate relationships among the study variables, and to make inferences regarding causality among the variables studied.

RESULTS

Plasma B-END values were obtained on 81 of 106 subjects enrolled in the study. Table I displays these data, subdivided by mean age (young: ≤ 29 years; older: > 29 years) and by natural killer cell activity (LNK: NK activity below the group mean at each of the three serial baseline assessments; normal: all others). We stratified by mean age and pattern of NK activity because previous research (10, 23) has shown that the pattern of NK activity profiles, rather than simple mean NK activity, has potentially important health relevance, and that a persistently low NK pattern is found more frequently in chronologically younger individuals. In general, plasma B-END values ranged between 5-10 pmol/l.

TABLE I

Plasma B-endorphin Levels in Normal Human Subjects
Stratified for Age and Natural Killer Cell Activity*

<u>Week of Study</u>	<u>Younger Subjects</u>		<u>Older Subjects</u>	
	<u>Low NK</u>	<u>Normal NK</u>	<u>Low NK</u>	<u>Normal NK</u>
	(pmol/l)			
Baseline 1	5.7 \pm 0.5	8.7 \pm 1.1	9.1 \pm 1.5	9.3 \pm 1.1
Baseline 2	6.8 \pm 0.6	8.1 \pm 1.0	9.4 \pm 1.3	7.5 \pm 0.7
Baseline 4	7.2 \pm 0.8	7.6 \pm 0.7	8.5 \pm 1.5	7.9 \pm 0.8
Follow-up 12	8.6 \pm 1.4	6.2 \pm 0.7	6.9 \pm 0.6	7.1 \pm 0.6
Follow-up 24	6.2 \pm 0.7	8.8 \pm 2.3	7.1 \pm 0.6	9.5 \pm 1.9

*Blood samples were drawn from 81 subjects during baseline weeks 1, 2 and 4, follow-up weeks 12 and 24. They were extracted and assayed for B-END as described in the methods section. Data are presented as means \pm sem.

We first carried out step-wise multiple regression analyses, predicting both overall morbidity, as well as upper respiratory morbidity, for the entire sub-sample and for the sample stratified into younger and older cohorts. For the entire sub-sample, 24% of overall morbidity variance could be accounted for by stress perception ($p < .0008$), age ($p < .01$), and the LNK pattern ($p < .05$), with an additional 2% of the variance being explained by the entrance of B-END values into the equation ($p < .1$). For upper respiratory morbidity, only age ($p < .001$) and stress perception ($p < .02$) entered the equation, accounting for 19% of the illness outcome variance.

When we stratified by age, for the older cohort, stress perception and chronological age significantly accounted for the two illness outcome variables. Specifically, 23% of overall morbidity variance was accounted for by stress perception ($p < .01$) and age ($p < .04$); 21% of URI variance was accounted for by age ($p < .02$) and marginally, by stress perception ($p < .06$). However, for the younger cohort, 29% of overall morbidity outcome variance was accounted for by stress perception ($p < .03$), B-END levels ($p < .04$), and the LNK pattern ($p < .02$) (8% of the variance being accounted for by B-END, alone); and for URI morbidity, 33% of the outcome variance was accounted for by age ($p < .01$), the LNK pattern ($p < .05$), B-END levels ($p < .08$) (B-END values accounting for an additional 6% of the variance), and marginally, by stress perception ($p < .1$). Thus, again, in the younger cohort, it appears that both the LNK pattern, and levels of plasma B-END might be playing a biologically important role in health status over the follow-up period.

Because findings from the main study (9,10) also showed that the LNK pattern and greater subsequent health risk were more prevalent in the younger sub-sample ($F=4.8$, $p < .005$), we therefore focused on this group ($N = 41$) in the analyses reported here. Table II presents values in the younger group for baseline NK activity (the average of the three sampling times) and follow-up morbidity. The data have been grouped according to normal and low-normal NK (LNK) activity. It is evident from the data in the table that all measures of NK function in the LNK group are significantly below those in the normal NK group. (It should be noted that this LNK pattern appears to be a "trait" like characteristic, as this pattern persisted for individuals within the LNK category, across the entire follow-up period for this study.) Plasma level for B-END (the average of all three baseline measurements), are also lower; simple

TABLE II

Baseline NK Activity and Plasma B-Endorphin Values,
and Follow-Up Morbidity Estimates in the Younger Subjects.

Parameter	Low NK	Normal NK Group	t	p
NK Activity				
Effector: Target Cell Ratio*				
50:1	26.95 \pm 1.91	48.08 \pm 1.83	5.6	.001
25:1	15.99 \pm 1.18	29.67 \pm 1.31	5.5	.001
12:1	9.63 \pm 0.86	18.59 \pm 1.19	4.4	.001
06:1	6.41 \pm 0.53	12.54 \pm 0.78	4.7	.001
Lytic Units	57.22 \pm 6.76	138.71 \pm 14.97	3.8	.001
B-END (pmol/l)+	6.48 \pm 0.41	8.15 \pm 0.64	2.2	.03
Morbidity (days)				
Overall	19.4 \pm 3.9	14.3 \pm 2.0	1.2	.2
URI	15.8 \pm 3.5	9.6 \pm 1.5	1.6	.1

data shown are means, \pm SEM

* % specific lysis

+ values are pooled from all three baseline measures

group means for overall and URI morbidity estimates during follow-up, however, were not significantly higher in the LNK group (Table II), though a trend was evident. This trend toward a higher morbidity in LNK activity and reduced plasma B-END levels was analyzed further statistically using a three-factor path model (9) developed by us, which was previously found to predict follow-up morbidity based on age, stress perception and LNK activity. Using this statistical model, we assessed specifically whether average baseline values of B-END affected URI via modulation of natural immunity or via some other mechanism. Table III presents the correlation matrix for variables in the model. A significant negative correlation was obtained between the LNK pattern and URI morbidity (i.e., LNK activity was associated with higher morbidity), and a significant positive correlation was observed between NK activity and B-END levels. As expected, a negative association was obtained between age and morbidity, and a positive association was seen between morbidity and perceived stress ("hassles").

TABLE III

Correlation Matrix of Path Model Variables Predicting
Upper Respiratory Infection (N = 41)

	<u>LNK</u>	<u>URI</u>	<u>AGE</u>	<u>Hassles</u>	<u>B-end</u>
LNK	1.000				
URI	-0.328*	1.000			
Age	0.183	-0.339*	1.000		
Hassles	-0.027	0.298*	-0.272	1.000	
B-end	0.375**	-0.180	0.204	-0.052	1.000

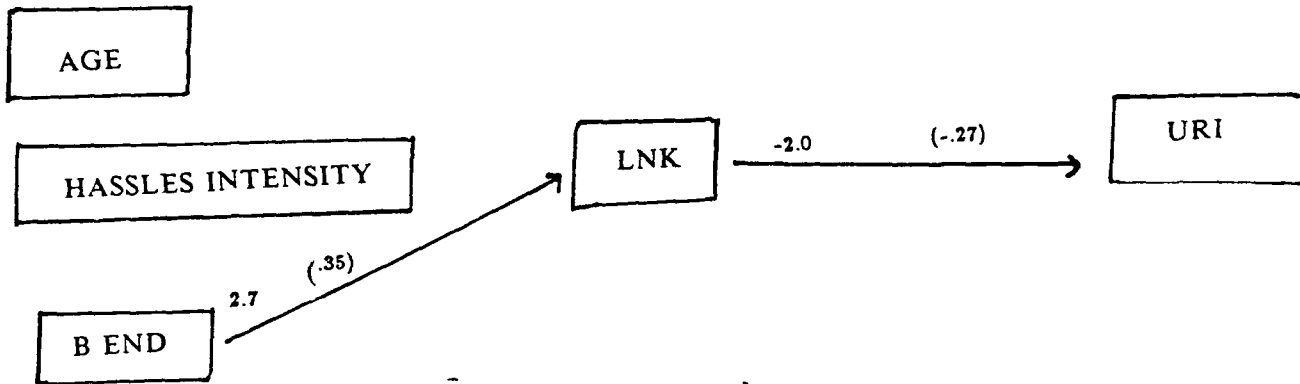
* p<.05

** p<.04

Figure 1 displays the path model that incorporates the baseline B-END findings. When baseline B-END values are entered along with age, perceived stress, and NK activity (LNK) as predictor variables, age and perceived stress did not emerge from the analysis as predictors of morbidity (illness), directly, or indirectly, acting via natural immunity. However, a statistically significant

FIG. 1

Path Analysis Model Predicting URI Morbidity
(Younger Cohort, $N=41$)



path was obtained linking baseline B-END levels with URI morbidity, via NK activity. That is, lower baseline levels of B-END predicted the persistently low NK activity pattern, which again predicted higher URI morbidity over the follow-up period. Statistical criteria for goodness-of-fit suggest that the data adequately fit the model tested (Chi-Square, NS; Goodness of Fit Index = 1; Root Mean Square residual = 0).

DISCUSSION

There are three main findings from the present study. First, it appears that persistently low NK activity (not abnormally low NK activity, but persistent activity at the lower end of normal functional values) is associated with greater infectious disease risk, particularly in younger individuals. Thus, as has been previously suggested (23), persistently low levels of NK cytotoxicity in a healthy population may place such individuals at higher risk for developing infectious illness over time.

Second, the role that "stress" might play in this regard appears to be more complex than has been assumed. Although in the main study findings, the report of perceived everyday stress directly predicted illness outcome, independently from immune variables, here, a "stress"-related neuropeptide, B-END, appeared to be associated with infectious morbidity via its potential effects on natural immunity. In fact, when B-END was entered, along with the psychosocial stress measure, into a multiple regression model predicting URI (not shown), the addition of B-END values reduced the significance of the psychosocial stress measure in accounting for illness outcome variance. The path model (Figure 1) also showed that entering B-END values into the equation eliminated the originally significant path linking reported distress with illness outcome.

Although there was no simple correlation in our sample between the stress measure scores and levels of plasma B-END ($r=.05$), there was a significant negative correlation between perceived stress and age ($r=-.27$), and a significant positive correlation between chronological age and levels of B-END ($r=.2$). Thus, age itself appears to play a major explanatory role in identifying populations at risk for infectious disease, at least when considering disease risk in those younger than age twenty-nine. Younger individuals reported more perceived stress, were more likely to show the LNK pattern, had generally lower levels of B-END concentration, and reported more infectious morbidity over the follow-up period.

There are at least two, potentially interrelated reasons why younger age might be associated with more infectious morbidity. First, young adults might have had less opportunity for prior infection by a range of infectious agents, and would thus have less pre-existing specific anti-viral immunity than older adults. In addition, younger adults might well have children at home who act as carriers for a variety of infectious illnesses, due to exposure to ill playmates and school peers. Thus, lack of prior exposure, coupled with current exposure to a variety of infectious agents, may place such younger individuals at increased risk for infectious morbidity. And second, younger individuals have not had the opportunity to develop a sense of perspective related to environmental stressors that age and accumulated "wisdom" might bestow on older individuals. Thus, perceived environmental stressors may be deemed more intense and stressful in those who have lived fewer years.

Third, the positive association between circulating B-END levels and NK activity, as well as the positive association between B-END and health, suggests that this peptide (and perhaps other endogenous opioids as well) might enhance immune function, including NK activity in humans, and act as a buffering agent related to stressful impingement. Indeed, B-END has been reported to increase immune functions of mononuclear cells in man and rat, and at the low concentrations that occur *in vivo* (2,3,24). For example, Van Epps and Saland (24) observed mononuclear cell chemotaxis to B-END in concentrations as low as 10^{-14} M. And, Mathews et al. (3) noted a significant augmentation of NK activity at these low B-END concentrations (i.e., 10^{-14} M). Further, Fiatarone et al. (25) demonstrated blockage of exercise induced enhancement of NK cytotoxicity by pre-exercise administration of the opiate antagonist naloxone. Of course, the changes we noted were small in comparison to those observed, for example, following the administration of potent pharmacologic agents (e.g., haloperidol [26]), or after strenuous exercise (27). But our study was designed as a prospective study on individuals living normally. The protocol was not intended to provide a specific stimulus of B-END release. Accordingly, it is not surprising to have found a much smaller variation in B-END levels associated with the differences in NK activity (Table II). Given the sensitivity of NK cells to B-END, it may be that these changes are biologically important. Because there are behavioral techniques that enhance NK activity (28,29) and increase circulating B-END levels (29), our findings suggest that such interventions might be considered to reduce health risk in young populations at risk for infectious diseases (e.g., military populations or medical students [30,31]).

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